Arcobacter thereius sp. nov., isolated from pigs and ducks

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During a Danish study on the prevalence of campylobacteria in pig abortions and food of animal origin, eight Gram-negative, slightly curved, rod-shaped, non-spore-forming bacteria were clustered by using amplified fragment length polymorphism analysis in a distinct phenon within the genus Arcobacter. In the present study, numerical analysis of whole-cell protein profiles also showed that all isolates clustered in a single group distinct from other recognized Arcobacter species. DNA–DNA hybridization among two representative strains exhibited a mean DNA–DNA relatedness value of 79 %. DNA–DNA hybridization with the type strains of recognized Arcobacter species revealed levels of DNA–DNA relatedness of 41 % or less. The DNA G + C content of the type strain was 28.5 mol%. Pairwise comparison of the 16S rRNA gene sequences with those of the type strains of established species identified Arcobacter cryaerophilus (97.9 %), Arcobacter cibarius (97.5 %) and Arcobacter skirrowii (97.2 %) as the nearest phylogenetic neighbours. The isolates could be distinguished from other Arcobacter species by means of the following biochemical tests: activities of catalase and urease, reduction of nitrate and growth on minimal medium, lack of growth at 37 °C under standardized aerobic and microaerobic conditions, in 4 % NaCl and 1 % glycine media. Finally, DNA fingerprints obtained by using enterobacterial repetitive intergenic consenus-PCR showed that the eight isolates represent eight strains of a single novel Arcobacter species, for which the name Arcobacter thereius sp. nov. is proposed. The type strain is LMG 24486T (=CCUG 56902T).

Slender, curved, aerotolerant Campylobacter-like bacteria were first isolated from the organs of aborted bovine and porcine fetuses (Ellis et al., 1977, 1978). The genus Arcobacter was described in 1991 as a second genus within the family Campylobacteraceae to encompass these bacteria that differ from campylobacters by their ability to grow at temperatures below 30 °C and their aerotolerance (Vandamme et al., 1991). At the time of writing, six species have been characterized: Arcobacter nitrofigilis, Arcobacter halophilus and a number of yet unclassified organisms, such as ‘Candidatus Arcobacter sulfidicus’, are environmental, which thus far have not yet been reported in human or animal specimens (Donachie et al., 2005; McClung et al., 1983; Wirsen et al., 2002). They have been recovered from diverse sources such as salt-water lakes, coastal seawater, water in underground cavities of oil wells, sediments in the Black Sea and various kinds of sludge.

The species Arcobacter butzleri, Arcobacter cryaerophilus and Arcobacter skirrowii are associated with reproduction

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA gene sequences of strains LMG 24486T and LMG 24487T, corresponding with strains 16389T and 16695-3 in the study by On et al. (2003), are AY314753 and AY314754, respectively. Accession numbers for the partial 23S rRNA gene sequences of the same strains are FM178224 and FM178225, respectively.

A dendrogram derived from numerical analysis of whole-cell protein profiles and DNA fingerprints obtained using enterobacterial repetitive intergenic consensus-PCR of A. thereius sp. nov. and Arcobacter reference strains are available as supplementary material with the online version of this paper.
disorders, mastitis and gastric ulcers in farm animals, but have also been isolated from healthy livestock (Van Driessche et al., 2003, 2004, 2005). A sixth species, *Arcobacter cibarius*, was isolated from chicken carcasses and piggery effluent environments (Chinivasagam et al., 2007; Houf et al., 2005).

Although *A. skirrowii* has been isolated recently from patients with enteritis (Samie et al., 2007; Wybo et al., 2004), *A. butzleri* and *A. cryaerophilus* are predominantly incriminated with enteritis and bacteremia in humans (Prouzet-Mauleon et al., 2006; Vandenbergh et al., 2004), but the mechanism of their pathogenicity has not been elucidated as yet (Houf & Stephan, 2007). In addition, the routes of infection are still unclear, but may include person-to-person contact and consumption of contaminated water and food. For that reason, arcobacters have been classified by the International Commission on Microbiological Specifications for Foods (ICMSF) as emerging pathogens (International Commission on Microbiological Specifications for Foods, 2002). Besides food and drinking water, close contact with pets has been identified as another potential infection source (Houf et al., 2008; Petersen et al., 2007).

In the present study, we report on the polyphasic taxonomic characterization of five *Arcobacter* isolates (11743–4, 11821–2, 16389T, 16695–3 and 16854–3) recovered previously from the liver and kidney of spontaneous porcine abortions, and three isolates (DU19, DU22 and DU3CL–2) from the cloacal content of ducks in Denmark (On et al., 2002, 2003). The isolates were recovered after a selective enrichment of the porcine tissues and the duck cloacal samples in Cefoperazone Amphotericin Teicoplanin (CAT) broth [Campylobacter enrichment basal medium (LAB 135, LAB M), 5 % (v/v) laked horse blood (Oxoid) and CAT selective supplement (Oxoid)] followed by inoculation on blood agar (blood agar base No. 2 plus 5 % defibrinated sheep blood; Oxoid) plates upon which a cellulose acetate filter (pore size 0.65 μm) was placed. All incubations were performed at 30 °C in a microaerobic atmosphere for at least 48 h (On et al., 2002).

Microaerobic subcultivation, by evacuating 80 % of the normal atmosphere and introducing a gas mixture of 8 % CO₂, 8 % H₂ and 84 % N₂ (v/v) into a jar, of the isolates on *Arcobacter* selective agar plates developed by Houf et al. (2001) at 28 °C showed colourless translucent small colonies and examination of these plates with Henry transillumination revealed that the colonies displayed the same characteristic bluish colour as reported previously for *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. cibarius* (Houf et al., 2008; Houf & Stephan, 2007).

Preparation of whole-cell proteins and sodium dodecyl sulphate PAGE were performed as described by Pot et al. (1994). The isolates were grown on Mueller–Hinton agar plates (CM0337; Oxoid) supplemented with 5 % (v/v) defibrinated horse blood (E&O Laboratories Ltd) and incubated microaerobically at 30 °C. Whole-cell protein profiles of *Arcobacter* reference strains and of reference strains of *Campylobacter* and *Helicobacter* species were available from previous studies (Houf et al., 2005; Vandamme et al., 1992). The densitometric analysis, normalization and interpolation of the protein profiles, and numerical analysis were performed using the GelCompar software package version 4.2 (Applied Maths). The similarity between all pairs of traces was expressed by the Pearson product moment correlation coefficient presented as percentages of similarity. A numerical analysis of the protein profiles of the eight isolates and of *Arcobacter* reference strains is shown in Supplementary Fig. S1 (available in IJSEM Online). All isolates grouped in a single cluster above a similarity level of 69 % and were clearly distinct from the other *Arcobacter* species.

DNA from the eight isolates was extracted by using the guanidinium thiocyanate method described by Pitcher et al. (1989). An expected *Arcobacter* genus-specific 1223 bp fragment was generated for all isolates in a genus-specific PCR assay (Harmon & Wesley, 1996). However, when using the *Arcobacter* species-specific multiplex-PCR assay of Houf et al. (2000), which targets a 257 bp fragment of the 23S rRNA gene of *A. cryaerophilus* (using the primers CRY1 and CRY2) and a 401 bp fragment of the 16S rRNA gene of *A. butzleri* and a 641 bp fragment of the 16S rRNA gene of *A. skirrowii*, all eight isolates produced the 257 bp of *A. cryaerophilus*. Therefore, the 23S rRNA gene sequences of the isolates LMG 24486T and LMG 24487 were determined. *In silico* annealing of the primer set CRY1 and CRY2 to the 23S rRNA genes of both isolates using FASTPCR software (Kalandar, 2007) revealed a difference of guanine instead of adenine at position one at the 3′-site with primer CRY1 and a cytosine instead of adenine at position 10 of primer CRY2. Both single differences explain the aspecific reaction of *A. thereius* sp. nov. in the multiplex PCR assay.

Differentiation by using numerical analysis of amplified fragment length polymorphism profiles using a *BglII–Csp6I*-based protocol used previously to characterize *Campylobacter* species clustered these isolates together in a distinct phenon from the other recognized *Arcobacter* species (On et al., 2003). Cluster analysis of the DNA-banding patterns obtained by using a modified enterobacterial repetitive intergenic consensus-PCR (Houf et al., 2002) revealed a large genotypic heterogeneity. Based on strain delineation as defined in previous studies (Houf et al., 2003; Van Driessche et al., 2004), eight genotypes could be identified (Supplementary Fig. S2, available in IJSEM Online).

Over 60 phenotypic characteristics were determined for the eight strains by use of an extensive biochemical identification scheme for arcobacters and related bacteria as described by On et al. (1996). These data were compared with the test results of extant species to assist in the evaluation of the taxonomic position of the novel species.
Arcobacter thereius isolated from pigs and ducks

(Houf et al., 2005; On et al., 1996, 2003). The strains gave results concordant with other Arcobacter species. All strains were Gram-negative, curved or weakly spiral rods under light microscopic examination. All strains were able to grow in aerobic and microaerobic conditions at 25 and 30 °C, but not at 37 °C. They produced oxidase and catalase, hydrolysed indoxyl acetate and reduced nitrate. Furthermore, strains were resistant to a range of antibiotics including cephalothin (32 mg l⁻¹), cefoperazone (64 mg l⁻¹) and carbenicillin (32 mg l⁻¹). Nonetheless, a critical comparison with test results of extant species demonstrated several phenotypic differences between all taxa. The most relevant phenotypic characteristics that differentiated the novel species from other taxa included in the genus are given in Table 1.

For electron microscopic analyses, strain LMG 24486ᵀ was grown on blood agar plates for 48 h at 28 °C under microaerobic conditions, and the bacteria were harvested and fixed in HEPES with 2.5 % glutaraldehyde for 24 h. Subsequently, the samples were post-fixed in 1 % osmium tetroxide for 2 h at room temperature. In the next step, the fixed samples were dehydrated through ascending grades of ethanol and transferred to a critical point drier CPD 030 (Bal-tec). The dried tissues were mounted on a metal stub and sputter-coated with platinum (JEOL JFC-1300 auto fine coat). Examination of the samples was performed on a JEOL JSM 5600 LV scanning electron microscope. Cells of strain LMG 24486ᵀ are slender rods, about 0.5 μm wide and 2.5 μm long with a single polar unsheathed flagellum at one end of the cell (Fig. 1).

Two strains, LMG 24486ᵀ (=16389ᵀ) and LMG 24487 (=16695-3) were selected for further genomic analysis. DNA–DNA hybridizations were performed with photo-biotin-labelled probes in microplate wells as described by Ezaki et al. (1989), using a HTS7000 Bio Assay Reader (Perkin-Elmer) for the fluorescence measurements. The hybridization temperature was 30 °C. DNA–DNA hybridization experiments showed that the representative strains exhibited a mean DNA–DNA relatedness of 79 %. Mean levels of DNA–DNA relatedness with the reference strains were 34 % with A. butzleri (LMG 10828ᵀ), 41 % with A. cryaerophilus (LMG 7536ᵀ), 32 % with A. skirrowii (LMG 6621ᵀ), 30 % with A. cibarius (LMG 21996ᵀ), 28 % with A. nitrofigilis (LMG 7604ᵀ) and 27 % with A. halophilus (ATCC BAA-1022ᵀ).

The DNA G+C contents of strains LMG 24486ᵀ and LMG 24487 were determined by enzymically degrading DNA into nucleosides as described by Mesbah & Whitman (1989). The nucleoside mixture obtained was then separated by using high-performance liquid chromatography with a Waters Symmetry Shield C8 column thermostated at 37 °C. The solvent used was 0.02 M NH₄H₂PO₄ (pH 4.0) with 1.5 % acetonitrile. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference. The DNA G+C contents of LMG 24486ᵀ and LMG 24487 were 28.5 and 28.6 mol%, respectively, confirming previously reported low DNA G+C values (between 27 and 30 mol%) for members of Campylobacteraceae (Vandamme et al., 1992).

To determine the phylogenetic position of the novel species, 16S rRNA gene sequences of strains LMG 24486ᵀ and LMG 24487 were determined as described previously (Vandamme et al., 2006). Sequences were assembled using BioNumerics v. 4.61 (Applied Maths), and aligned using

Table 1. Characteristics that differentiate Arcobacter thereius sp. nov. from other members of the genus

<table>
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<tr>
<th>Characteristic</th>
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Comparison by using the FASTA algorithm to the EMBL sequence database revealed that the nearest phylogenetic neighbours were *A. cryaerophilus* (97.9%), *A. cibarius* (97.5%) and *A. skirrowii* (97.2%). Clustering was performed by using neighbour-joining (Saitou & Nei, 1987) with the BioNumerics v. 4.61 software package. Unknown bases were discarded for the analysis, and bootstrap values were determined using 500 replicates (Fig. 2).

The pathogenic role of *A. thereius* sp. nov. in porcine reproduction abnormalities is unknown, and the high prevalence of arcobacters in pig abortions, for which no other established abortifacient agent was detected, also needs further attention. In particular, the role of individual *Arcobacter* species needs further study as many have been isolated from sows, boars and piglets with neither health nor reproduction problems in the herd. Moreover, multiple species such as *A. butzleri*, *A. skirrowii* and *A.

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**Fig. 1.** Scanning electron micrograph of a cell of strain LMG 24486T (*A. thereius* sp. nov.). Bar, 1 μm.

**Fig. 2.** Phylogenetic tree of nine *Arcobacter* strains based on 16S rRNA gene sequence similarity data. Clustering was performed by using the neighbour-joining method. Numbers at branch points indicate the number of times out of 100 that the clade was recovered by bootstrap resampling (number of bootstraps, 500). Bar, 1 % sequence divergence.
Arcobacter thereius have been recovered from aborted fetuses derived from a single sow (On et al., 2002). A reliable and fast identification method should be developed as, at present, delineation of the different species is most efficiently established by using protein analysis or amplified fragment length polymorphism, which are both not routinely applied. The PCR-based methods currently applied generate no amplicons for A. thereius sp. nov. and A. cibarius (Houf et al., 2000; Kabeya et al., 2003) or, as with the m-PCR by Houf et al. (2000), misidentify A. thereius sp. nov. as A. cryaerophilus. The lack of growth at 37 °C under conditions that cultivate other host-associated arcobacters is noteworthy, especially as it is associated with warm-blooded animals such as pigs and especially ducks. An environmental contamination, in particular in the case of birds, cannot be totally excluded. The presence of A. thereius sp. nov. in food animals and the potential as a zoonotic pathogen requires further study.

Very recently, a novel species, Arcobacter mytili sp. nov., has been published (Collado et al., 2009). Isolates have been recovered from mussels and sequence analysis of the 16S rRNA and rpoB genes showed a new lineage within the genus. A. mytili sp. nov. can be differentiated from A. thereius sp. nov. by the lack of hydrolysis of indoxyl acetate, nitrate reduction and susceptibility to cepofenoxazone.

**Description of Arcobacter thereius** *sp. nov.*

_Arcobacter thereius_ [the're'ı.us. N.L. masc. adj. (from Gr. adj. thēreioς) pertaining to an animal].

Cells are slightly curved, Gram-negative rods, 2.5 μm long and 0.5 μm wide. Form whitish, low-convex, non-swarming, smooth-rounded colonies with entire margins of about 2 mm in diameter on blood agar after 72 h incubation at 28 °C under microaerobic conditions. Forms translucent to opaque smooth-rounded colonies of 1–2 mm diameter on _Arcobacter_ selective agar. In a microaerobic atmosphere, growth is observed at room temperature (18–22 °C) and 30 °C, but not at 37 °C after 3 days incubation. No growth occurs at 37 °C in aerobic conditions; growth occurs at 25 and 30 °C aerobically. Produces oxidase and catalase, hydrolyses indoxyl acetate and reduces nitrate. Alkaline phosphatase, urease, DNAse and hippuricase activities are not detected, and selenite is not reduced. Hydrogen sulfide is not produced in triple-sugar iron agar medium. Under microaerobic conditions, grows on non-supplemented minimal and potato starch media, and on media containing 1.0 % glycine, 32 mg cephalothin l⁻¹, 64 mg cepofenoxazone l⁻¹, 0.05 % sodium fluoride, 0.001 % sodium arsenite and 0.032 % methyl orange. Does not grow on casein, tyrosine, 0.02 % pyronein media or media containing 4.0 % NaCl. Growth on MacConkey, lecithin and _Campephobacter_ charcoal-deoxycholate base media, and on media containing 0.04 % triphenyl-tetrzolium chloride, 0.1 % potassium permanganate, basic fuchsin, Crystal violet or Janus green is strain dependent. Haemolysis on blood agar and growth on a blood agar medium containing 32 mg nalidixic acid l⁻¹ is strain dependent. Growth under anaerobic conditions on both non-supplemented 5 % blood agar and blood agar containing 0.1 % trimethylamine N-oxide is variable. Grows on non-supplemented nutrient agar, and on media containing 2.0 % NaCl, 0.05 % safranin and 32 mg carbenicillin l⁻¹.

The type strain, LMG 24486T (=CCUG 56902T), was isolated from the kidney and liver of an aborted pig foetus, in Denmark in 1996. Strain LMG 24487 is a reference strain.

**References**


